PB86-190121

Metabolism of 1-Nitropyrene by Cultured Rabbit Alveolar Macrophages and Respiratory Tract Tissues

(U.S.) Health Effects Research Lab
Research Triangle Park, NC

1986

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completi

1. REPORT NO.	2.	3. PB86-190121
EPA/600/J-86/032		

4. TITLE AND SUBTITLE	5. REPORT DATE
Metabolism of 1-Nitropyrene by Cultured Rabbit Alveolar Macrophages and Respiratory Tract Tissues (Journal Version)	1986
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
Leon C. King, Louise M. Ball, Marcus Jackson, Jeff P. Inmon, and Joellen Lewtas	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
USEPA, Health Effects Research Laboratory, Research Triangle Park, NC 27711 / Northrop Services, Inc., Research Triangle Park, NC 27709	11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
USEPA, Health Effects Research Laboratory, Research Triangle Park, NC 27711 / Northrop Services, Inc., Research Triangle Park, NC 27709	Journal Article
	14. SPONSORING AGENCY CODE
	EPA/600/11

15. SUPPLEMENTARY NOTES

Toxicology and Applied Pharmacology 82, 292-300 (1986) February No. 2

16. ABSTRACT The metabolism of 1-nitropyrene (1-NP) was studied in cultured rabbit alveolar macrophages and respiratory tissues from both lung and trachea. Metabolites from the incubation medium and from the macrophages and respiratory tissues were isolated and quantitated by HPLC. The following metabolites were isolated and quantitated: 1-nitropyrene-4,5- or -9,10-dihydrodiol (K-DHD), N-acetyl-1-aminopyrene (NAAP), 1-aminopyrene (1-AMP), 10-hydroxy-1-nitropyrene (10-OH-1-NP), 4-,5-,6-,8- or 9-monohydroxy-1-nitropyrene (phenols), and 3-hydroxy-1-nitropyrene (3-OH-1-NP). The total percentage of 1-NP metabolism was significantly greater in the lung and tracheal tissue (28.0 to 23.0% of the recovered ^{14}C, respectively) compared to the alveolar macrophages (6.3%). The tracheal tissue was found to have the highest activity both in 1-NP metabolism and intracellular metabolite concentration. A major portion of the 1-NP metabolites produced were released into the incubation medium. The majority of the metabolites produced by tracheal and lung tissue, 66 and 80% respectively, were ethyl acetate-extractable. Macrophages, however, produced and released mostly water-soluble metabolites. The metabolites retained within the cells or tissues were predominantly ethyl acetate-extractable rather than water soluble (5:1 for the macrophages and trachea, 17:1 for the lung tissue). The metabolite profiles obtained demonstrate that metabolism by both nitro-reduction and ring oxidation occurs in respiratory tissue, and a degree of tissue specificity in the formation of metabolites exists. Very little ring oxidation occurred in the macrophages but was demonstrated in the lung and tracheal tissue.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
Release to Public	Unclassified	10
	20. SECURITY CLASS (This page) Unclassified	22. PRICE

EPA/600/J-86/032
JOURNAL ARTICLE

TOXICOLOGY AND APPLIED PHARMACOLOGY 82, 292–300 (1986)

Metabolism of 1-Nitropyrene by Cultured Rabbit Alveolar Macrophages and Respiratory Tract Tissues

LEON C. KING, LOUISE M. BALL,[1] MARCUS JACKSON,[2]
JEFF P. INMON, AND JOELLEN LEWTAS[3]

Genetic Toxicology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711

Received May 18, 1985; accepted September 18, 1985

Metabolism of 1-Nitropyrene by Cultured Rabbit Alveolar Macrophages and Respiratory Tract Tissues. KING, L. C., BALL, L. M., JACKSON, M., INMON, J. P., AND LEWTAS, J. (1986). *Toxicol. Appl. Pharmacol.* **82**, 292–300. The metabolism of 1-nitro[^{14}C]pyrene (^{14}C-1-NP; 8.1 μM) was studied in cultured (20 hr) rabbit alveolar macrophages, lung tissue, and tracheal tissue. Metabolites from the incubation medium and from the macrophages and respiratory tract tissues were extracted and then analyzed and quantified by high-pressure liquid chromatography. The following metabolites were detected in the lung and tracheal tissue incubation medium: 1-nitropyrene-4,5-dihydrodiol, *N*-acetyl-1-aminopyrene, 1-aminopyrene, and 10-hydroxy-1-nitropyrene. Nitropyrene phenols (4-, 5-, 6-, 8- or 9-hydroxy-1-nitropyrene) and 3-hydroxy-1-nitropyrene were only detected in the lung and tracheal tissue and not in the incubation medium for these tissues. Minor amounts of 1-aminopyrene and 10-hydroxy-1-nitropyrene were detected in the macrophage incubation medium, and only minute quantities of 1-nitropyrene-4,5-dihydrodiol, 1-aminopyrene, and 10-hydroxy-1-nitropyrene were detected in macrophages. The total percentage of 1-NP metabolism was significantly greater in the lung and tracheal tissue (28.0 and 23.0% of the recovered ^{14}C, respectively) than in the alveolar macrophages (6.3% of the recovered ^{14}C). The tracheal tissue was found to have the highest activity both in 1-NP metabolism and intracellular metabolite concentration. A major portion of the 1-NP metabolites produced was released into the incubation medium. The majority of the metabolites produced by tracheal and lung tissue, 70 and 84%, respectively, were ethyl acetate extractable. The metabolites retained within the cells or tissues were also predominantly ethyl acetate extractable rather than water soluble (83% for the macrophages and trachea, 95% for the lung tissue). The metabolite profiles obtained demonstrate that metabolism by both nitro reduction and ring oxidation occurs in respiratory tissue, and a degree of tissue specificity in the formation of metabolites exists. Ring oxidation was demonstrated in the lung and tracheal tissue, but very little occurred in the macrophages. © 1986 Academic Press, Inc.

Several *in vivo* studies have shown that inhaled diesel particles generated by automotive engines are readily deposited in the respiratory tract (Chen *et al.*, 1980; Chan *et al.*, 1981; White and Garg, 1982; McClellan *et al.*, 1982; Vostal *et al.*, 1982a,b), especially in the alveolar macrophages (White and Garg, 1982; McClellan *et al.*, 1982; Rudd and Strom, 1981). Some of these particles are cleared rapidly from the lung to the gastrointestinal tract, but a substantial portion are retained in the lung for a very long period of time (Chan *et al.*, 1981; Vostal *et al.*, 1982a; Rudd and Strom, 1981). Diesel particles are emitted as submicron chain aggregates of primary par-

[1] Present address: Department of Environmental Sciences and Engineering, Rosenau Hall, 201 H, University of North Carolina, Chapel Hill, North Carolina 27514.

[2] Present address: Environmental Health Research and Testing, Inc., P.O. Box 12199, Research Triangle Park, N.C. 27709.

[3] To whom reprint requests should be addressed.

0041-008X/86 $3.00
Copyright © 1986 by Academic Press, Inc.
All rights of reproduction in any form reserved.
NTIS is authorized to reproduce and sell this report. Permission for further reproduction must be obtained from the copyright owner.

ticles that have a carbonaceous core and absorbed organic components (Vostal *et al.*, 1982a). These absorbed organic components are mutagenic in many short-term bioassays and tumorigenic in SENCAR mice (Lewtas, 1982; Nesnow *et al.*, 1982). Nitro-substituted polycyclic aromatic hydrocarbons (NO_2-PAHs), including the bacterial mutagen 1-nitropyrene (1-NP) (Mermelstein *et al.*, 1981), have been identified and quantified in diesel particle extracts (Peterson *et al.*, 1981; Schuetzle *et al.*, 1981; Nishioka *et al.*, 1982) at concentrations that account for up to 30% of the mutagenicity of these extracts (Nishioka *et al.*, 1982; Schuetzle, 1983). Several NO_2PAHs (e.g., 1-NP) have been shown to be mutagenic in mammalian cells (Lewtas, 1982; Cole *et al.*, 1982; Campbell *et al.*, 1981) and carcinogenic in rodents (Hirose *et al.*, 1984).

The prolonged retention of diesel particles by the lung ensures the exposure of respiratory tissues to these mutagens and potential carcinogens. Diesel particles incubated with rabbit pulmonary macrophages were engulfed and lost both mutagenic activity (62 to 92%) and 1-NP (22 to 24%) (King, 1983b). These results suggested that lung macrophages can metabolize mutagenic nitroaromatics such as 1-NP found on diesel particles and prompted us to investigate the role of respiratory tract tissues that might be similarly exposed. We therefore initiated a series of *in vitro* studies to characterize the metabolism and activation of 1-NP in pulmonary tissues; the rabbit lung was utilized as a model system because of its susceptibility to chemical carcinogenesis (Hirao *et al.*, 1972) and the quantity of tissues available for isolation.

We have demonstrated that macrophages and respiratory tract tissues can activate 1-nitro[^{14}C]pyrene (^{14}C-1-NP) to intermediates that bind to protein and DNA (King *et al.*, 1983a). 1-Nitropyrene is metabolized by the liver *in vitro* (El-Bayoumy and Hecht, 1983; Ball *et al.*, 1984a; Bond *et al.*, 1984b; Howard *et al.*, 1985) to phenols and *in vivo* by the rat to *N*-acetyl-1-aminopyrenols (NAAP) (Ball *et al.*, 1984b), which were more mutagenic to *Salmonella* TA98 than the parent compound. This paper reports the comparative metabolism of 1-NP in rabbit alveolar macrophages, lung tissues, and tracheal tissues.

METHODS

Animals. New Zealand white male rabbits (60 days old) weighing 1.5 to 2.0 kg (Dutchland Laboratory Animals, Denver, Pa.) were individually housed in a 12-hr light/dark cycle animal room, maintained on antibiotic-free Purina Rabbit Chow (Ralston Purina, St. Louis, Mo.), and provided water *ad libitum*.

Chemicals. 1-Nitro[4,5,9,10-$^{14}C_4$]pyrene (^{14}C-1-NP; 59.5 mCi/mmol) prepared by nitration of [4-^{14}C]pyrene, unlabeled 1-NP, 1-nitro[*cis*]-4,5-dihydro-4,5-dihydroxypyrene (1-NP-diol), and the isomers 3-, 4-, 5-, 6-, 8-, 9-, and 10-hydroxy-1-NP were synthesized and characterized by Mid-West Research Institute (Kansas City, Mo.), under EPA Contract 68-02-3177. Structural assignments were confirmed by proton nuclear magnetic resonance (NMR) (Ball *et al.*, 1984b; unpublished results). 1-Aminopyrene (1-AMP; Grade II) was purchased from Sigma Chemical Company (St. Louis, Mo.). *N*-Acetyl-1-aminopyrene was prepared by acetylation of 1-AMP (dissolved in dimethyl sulfoxide (DMSO)) with acetic anhydride and recrystallization from aqueous ethanol. These compounds are described more fully elsewhere (Ball *et al.*, 1984a,b). Unlabeled 1-NP was added to ^{14}C-1-NP to adjust its specific activity to 10 mCi/mmol.

Cell and tissue preparation. Rabbit alveolar macrophages (RAM) were obtained by lung lavage (Garrett *et al.*, 1981). Immediately after the lung lavage (six to eight rabbits), the tracheas were removed and placed in a beaker containing 200 ml of medium M-199 (Gibco, Grand Island, N.Y.) on ice at 4°C. Residual connective tissue or muscle was removed, the tracheas were sliced longitudinally into strips, and each strip was cut into 1-mm^3 sections. The sections were washed twice with physiological saline and weighed. The average individual tracheal tissue weights were 5.8 ± 0.1 g (wet weight).

Rabbit lungs (two animals/experiment) were perfused as described below and then removed. The animals were anesthetized by a simultaneous injection of 150 mg of sodium pentobarbital and 1000 units of sodium heparin into the marginal ear vein. The animals were shaved from the mandible to midabdomen, the shaved area was washed with 70% ethanol, and a midline incision was made from the mandible to just below the sternum. The sternum was split with a scalpel and bone cutters from the manubrium to the xiphoid process, and the ribs were retracted with a self-restraining retractor. The thymus and pericardium were removed and a cannula was inserted into the pulmonary artery and secured with a ligature. The lungs were then perfused for 10 min at a rate of 25 ml/min with 250 ml of a chelating solution of 0.5 mM ethylene glycol bis(β-

aminoethyl ether)-*N,N'*-tetraacetic acid (Sigma Chemical Co.) in Hanks' Ca^{2+}- and Mg^{2+}-free balanced salt solution supplemented with 10 mM Hepes buffer and 0.05 mg/ml gentamicin (Schering Corp., Kenilworth, N.J.). The aorta and dorsal vena cava were immediately severed for drainage. The perfusion solution was maintained at 37°C in a water bath and continuously equilibrated with 5% CO_2 and air throughout the procedure. The lungs were thoroughly blanched, removed, separated from the vascular tissue, and placed in a beaker containing 200 ml of medium M-199 on ice at 4°C. The lung tissue was cut into 1-mm^3 pieces and the total minced tissue was weighed. The average individual lung tissue weights were 12.6 ± 0.2 g (wet weight).

Cell culture treatments and separations. Macrophages (0.5×10^6 cells/ml), lung tissue (31.0 ± 4.0 mg/ml), and tracheal tissue (14.0 ± 0.4 mg/ml) were separately incubated in seven 150-cm^2 flasks (total volume of 30 ml/flask) for 20 hr at 37°C under a humidified atmosphere of 5% CO_2 in air. The tissue culture medium M-199 contained 10% heat-deactivated fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and kanamycin (100 μg/ml). 1-Nitro[^{14}C]pyrene dissolved in DMSO (Burdick and Jackson Laboratories, Inc., Muskegon, Mich.) was added at a final concentration of 8.1 μM. The experiment was repeated three times. A control sample containing ^{14}C-1-NP (8.1 μM; 81.0 nCi/ml) and medium was incubated for 20 hr without cells or tissues. Dye-exclusion tests with trypan blue indicated that the RAM cultures contained 95.8 ± 1.6% viable cells after 20 hr.

After incubation, the control medium was decanted, extracted, concentrated, and analyzed by high-pressure liquid chromatography (HPLC). The macrophage medium including unattached cells was decanted and centrifuged at 300*g* to pellet unattached macrophages. The flasks were washed twice with 5 ml of phosphate-buffered saline (PBS), and this wash was added to the decanted medium. Macrophages were detached from the culture flasks by adding 2 ml of 0.25% trypsin, resuspended in 10 ml of the decanted medium, and centrifuged at 300*g* for 15 min. The supernatant fluid was added to the decanted medium, and the macrophages were added to the previously pelleted unattached macrophages. The lung and tracheal tissues were separated from their incubation medium by filtration through 62- and 250-μm nylon mesh filters (Small Parts, Inc., Miami, Fla.).

Extraction of metabolites. The exposure medium (100 ml) from macrophages, lung tissue, or tracheal tissue was extracted three times with three volumes of cold methylene chloride (DCM):methanol (MeOH) (1:1 v/v, DCM:MeOH) and centrifuged at 500*g* for 15 min. The protein precipitate from this centrifugate was extracted twice with 10 ml of cold DCM:MeOH. All extracts were pooled, and then the solvent was removed by rotary evaporation at 37°C.

The macrophages were lysed with 25 ml of lysing buffer (50 mM Tris–HCl:10 mM EDTA, 1% sodium dodecyl sulfate [SDS], pH 8.0), and the tissues were homogenized in 20 ml of the lysing buffer. Macrophage lysate (5 ml), lung tissue homogenate (10 ml), or tracheal tissue homogenate (10 ml) was extracted as described above. Dried residues of tissue or medium extracts were resuspended in 50% aqueous MeOH for direct HPLC analysis or in 5 mM phosphate buffer for enzyme hydrolysis. Control samples were also extracted by this procedure. Radioactivity recoveries for ^{14}C-1-NP in the control samples were greater than 95%.

Enzyme hydrolysis. The dried DCM:MeOH extracts were resuspended in 30 ml of 5 mM phosphate buffer (pH 6.9) and incubated for 4 hr at 37°C with aryl sulfatase (5 units) or β-glucuronidase (1000 units, Sigma Type VII from *Escherichia coli*). The incubation mixture was extracted three times with ethyl acetate (EA) and the extract was rotary evaporated to dryness at 37°C. This residue was resuspended in 50% aqueous MeOH for HPLC analysis. Over 95% of the total ^{14}C was recovered in the aqueous MeOH. After the EA extraction, 5% of the total ^{14}C remained in the aqueous phase.

Quantitation of radioactivity. Radioactivity was determined by liquid scintillation counting (Model 460C, Packard Instrument Co., Downers Grove, Ill.). Efficiency of counting was calculated from quenching of an external standard and a previously established calibration curve.

Quantitation of DNA. DNA and protein were isolated and quantified as described previously (King *et al.,* 1983a).

Analysis of 1-NP metabolites. 1-Nitropyrene metabolites were analyzed with a Varian Model 5060 high-pressure liquid chromatograph equipped with a Model CDS 401 Vista Data System (Varian Associates, Inc., Walnut Creek, Calif.). The columns used for analysis were a Zorbax ODS column (9.4 mm × 25 cm, E.I. duPont de Nemours Co., Wilmington, Del.) and a guard column (4.6 mm × 5 cm) packed with pellicular ODS (Whatman, Inc., Clifton, N.J.). The metabolites were eluted with a MeOH:water gradient (60 to 100%, 2%/min) for 60 min at a flow rate of 1.5 ml/min. The eluate was monitored with a Varian Model 50 uv-vis detector at 280 nm and a Model MPF 2A fluorometer (Perkin Elmer Corp., Norwalk, Conn.; excitation, 360 nm; emission, 430 nm). A Gilson Model B-200 collector (Gilson Medical Electronics, Middleton, Wisc.) was used to collect fractions directly into scintillation vials.

Aliquots (100 μl) of each fraction were sampled to determine ^{14}C content for metabolite quantitation. Metabolites were identified by comparing their spectral properties (uv and/or fluorescence) and chromatographic characteristics (retention time) with those of the standard reference compounds. The synthesis, isolation, and characterization of these reference compounds have been described in detail elsewhere (Ball *et al.,* 1984b). The reference compounds and metabolites eluted are indicated in Fig. 1. The 1-NP-diol metabolite produced by lung and tracheal tissue was isolated and analyzed by uv spectrometry (Beckman Model 25, Beckman Instrument Co., Fullerton, Calif.). The synthetic standard *cis*-4,5-dihydro-4,5-dihydroxy-1-nitropyr-

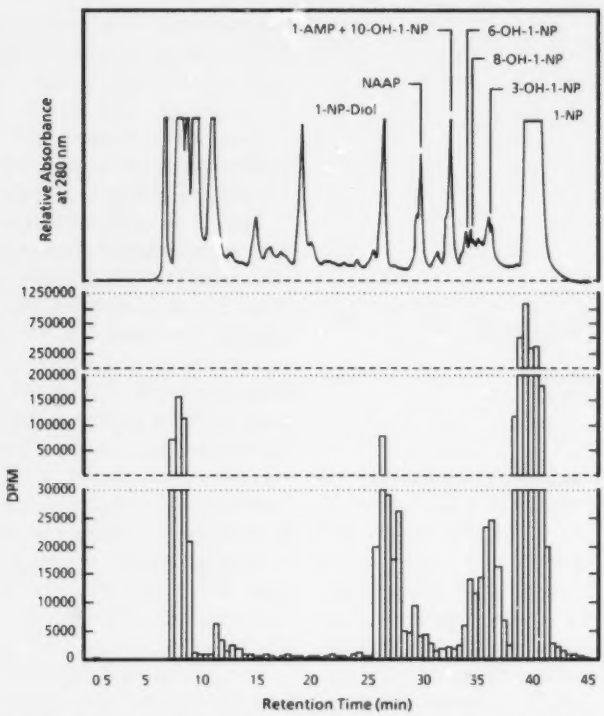

FIG. 1. HPLC profile of 1-NP metabolites from lung medium coeluted with unlabeled reference standard compounds. Lung tissue (31 mg/ml) was incubated in seven 150-cm^2 flasks (total vol 30 ml/flask) for 20 hr at 37°C under a humidified atmosphere of 5% CO_2 in air. The lung tissue medium was extracted three times with three volumes of cold methylene chloride:methanol (1:1 v/v) and the solvent was removed by rotary evaporation at 37°C. The residue was resuspended in aqueous methanol and spiked with synthetic standard compounds before HPLC analysis. The upper panel represents the relative absorbance at 280 nm for the metabolites and reference standard metabolites and the lower panel is indicative of the disintegrations per minute associated with the metabolites isolated from lung medium.

ene was used as a chromatographic marker because it was more accessible by chemical synthesis than the *trans* isomer. Figure 2 shows the uv spectrum of the metabolite that coeluted with this marker. The spectrum is virtually indistinguishable from that of the *cis* isomer (also shown in Fig. 2) and also from that of the metabolite identified as the *trans* 4,5-diol by El-Bayoumy and Hecht (1983). The metabolite decomposed to material chromatographing as nitropyrenols both spontaneously (on occasion) and on treatment with acid. Furthermore, the aromatic proton region of the proton NMR spectrum of this metabolite corresponds to that of the synthetic *cis* (Ball *et al.*, 1984b); the benzylic proton region of the NMR spectrum was not unambiguously interpretable, therefore we could not determine that coupling constant that would allow definite assignation of the metabolite structure. The weight of evidence therefore indicates that the metabolite is indeed a 4,5-dihydro-4,5-dihydroxy-1-nitropyrene by analogy with other PAHs and the work of El-Bayoumy and Hecht (1983). The 6- or 8-hydroxy-1-NP isomers chromatographed together and were designated phenols. 1-Aminopyrene and 10-hydroxy-1-NP (10-OH-1-NP) coeluted in this system. Other material that did not correspond chromatographically or spectroscopically with the synthetic reference compounds eluted at the solvent front (designated SF) or shortly afterward (polar unknown metabolites).

RESULTS

Initial time-course studies with macrophages showed that before 12 hr little or no metabolism was detectable. At 12 hr, metab-

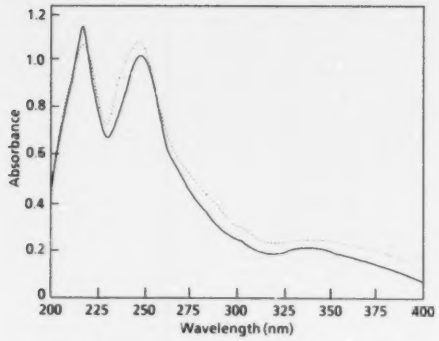

FIG. 2. Ultraviolet spectrum of biological 1-NP-diol (· · ·) and synthetic 1-NP-diol (—).

olism was clearly detectable, and the maximum extent of transformation was seen at 24 hr (6.8%). Therefore, 20 hr was selected as a suitable incubation time, and this time had been used in a previous study (King *et al.*, 1983a). Cytotoxicity studies (trypan blue exclusion and cellular adenosine triphosphate (ATP) content of rabbit alveolar macrophages exposed to 1-NP (1 to 100 μg/ml)) indicated little cytotoxicity up to 50 μg/ml (data not shown). The dose (2 μg/ml) was selected for compatibility with previous studies that utilized 1 mg/ml of diesel (Nissan) particle extracts containing 1.9 μg 1-NP/mg (unpublished results). The *in vivo* ratio of macrophages, lung tissues, and tracheal tissue determined to be present upon isolation was maintained throughout these cultures.

The ^{14}C recovered after incubation had undergone substantial metabolism, which varied both quantitatively (Table 1) and qualitatively (Figs. 3 and 4) between the different tissues studied. The extent of 1-NP biotransformation was greater in the lung and tracheal tissues (28.0 and 23.0% of recovered ^{14}C, respectively) than in the alveolar macrophages (6.3%). In the medium incubated without cells or tissues, less than 2% of the ^{14}C-1-NP had undergone transformation (data not shown). Relative to tissue protein content, the trachea was found to have the highest activity both in total 1-NP metabolism and in intracellular metabolite concentration. Macrophages, lung tissue, and tracheal tissue released most of the metabolites produced into the incubation medium; the trachea was the most active in this respect. The extent of sulfate and glucuronide conjugation was evaluated by comparing the profiles

TABLE 1

METABOLISM OF 1-NITROPYRENE BY MACROPHAGES, LUNG TISSUES, AND TRACHEAL TISSUES IN CULTURE[a]

	Metabolites in medium (nmol/mg protein)			Cellular metabolites (nmol/mg protein)			
	Total metabolites in medium (nmol)	Ethyl acetate extractable	Water soluble	Total cellular metabolites (nmol)	Ethyl acetate extractable	Water soluble	Total metabolism[b] (%)
Macrophages	17.0	1.3 ± 0.2	0.3 ± 0.1	11.4	0.9 ± 0.3	0.2 ± 0.1	6.3
Lung tissue	249.0	2.3 ± 0.6	0.6 ± 0.4	120.0	1.3 ± 0.4	0.1 ± 0.01	28.0
Tracheal tissue	420.0	13.3 ± 3.10	7.0 ± 0.1	99.0	4.0 ± 2.0	1.0 ± 0.4	23.0

[a] Rabbit alveolar macrophages, lung tissues, and tracheal tissues were incubated with ^{14}C-1-NP for 20 hr. The macrophages were lysed and the lung and tracheal tissue homogenized separately in lysing buffer. Protein analysis was performed on an aliquot of each sample. Reaction products from the remaining aliquots were extracted with DCM: MeOH, rotary evaporated to dryness, reconstituted in 0.005 M phosphate buffer, pH 6.9, and incubated with β-glucuronidase. Reaction products were extracted again with ethyl acetate (EA), then separated from unchanged 1-NP, and quantified by HPLC and liquid scintillation counting. Water-soluble metabolites were quantified from the ^{14}C remaining in the aqueous phase. The results are the mean of three separate experiments (±SE).

[b] Calculation based on nanomoles of metabolites produced relative to the total unchanged 1-NP recovered.

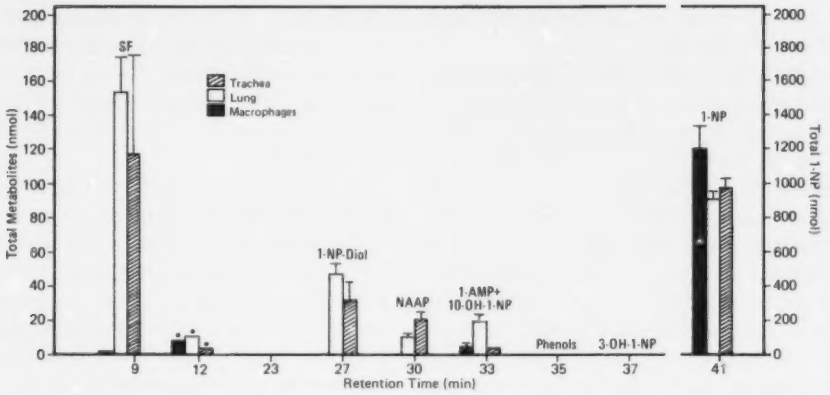

FIG. 3. Quantity of 1-NP metabolites and unaltered 1-NP in the culture medium of macrophages, lung tissues, and tracheal tissues. Exposure conditions and isolation of metabolites are described under Methods. The bars represent the mean total amount (nmol) of metabolites ± SE from three separate experiments. Asterisk indicates metabolites appearing in only one experiment.

of both intracellular and extracellular metabolites of macrophages, lung, and tracheal tissue before and after incubation with sulfatase or β-glucuronidase. There was no difference between the metabolite profile of the β-glucuronidase or sulfatase treatments when compared to the nonenzymatic treatment (data not shown). The metabolites retained within the cells or tissues were predominantly EA extractable (83% for macrophages and trachea, 95% for lung tissue). The majority of the metabolites released into the medium were also EA extractable (67% for tracheal tissue and 80% for lung tissue and macrophages) and were overall the major metabolite group formed by these cells and tissues. The tracheal tissue was the most active in production and release of water-soluble material, which represented 33% of the metabolites formed by these cells and released into the medium. The

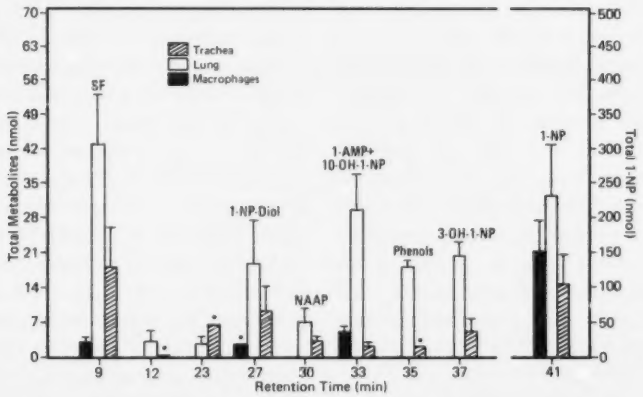

FIG. 4. Quantity of 1-NP metabolites and unaltered 1-NP in macrophages, lung tissues, and tracheal tissues. Exposure conditions and isolation of metabolites are described under Methods. The bars represent the mean total amount (nmol) of metabolites ± SE from three separate experiments. Asterisk indicates metabolites appearing in only one experiment.

unextractable water-soluble metabolites were not further analyzed.

The EA-extractable metabolites (which included the aglycones of hydrolyzed glucuronides) were analyzed and quantified by HPLC and liquid scintillation counting. Figure 3 represents the distribution of ^{14}C-1-NP and its metabolites in medium from the lung, trachea, and macrophage incubations. The majority of the metabolites eluted at the solvent front. Other metabolites identified in the medium included 1-NP-diol, NAAP, 1-AMP, and 10-OH-1-NP. Minor amounts of a polar metabolite eluting at 12 min were also detected. The medium from the macrophages contained only minor amounts of 1-AMP and 10-OH-1-NP; the tracheal and lung medium contained significant quantities of 1-NP-diol and NAAP. Comparisons between the quantities of lung and tracheal tissue metabolites revealed no significant differences. However, the macrophage incubations resulted in significantly lesser amounts of all metabolites, and no metabolites were detected at the solvent front.

The intracellular metabolites in the lung and tracheal tissues (Fig. 4) were qualitatively similar. The major metabolites in lung tissues were 4-, 5-, 6-, 8-, or 9-hydroxy-1-NP (phenols), 1-AMP, 1-NP-diol, and metabolites at the solvent front. Lesser amounts of NAAP and polar metabolites eluting at 12 and 23 min were detected in lung tissues. Tracheal tissues showed a similar metabolite profile with proportionately less phenols and 1-AMP. In macrophages, the only metabolites detected were 1-AMP + 10-OH-1-NP, 1-NP-diol, and relatively minor amounts of metabolites at the solvent front. Unmetabolized ^{14}C-1-NP amounts in the macrophages and respiratory tissues were similar. However, the incubation medium from lung tissue, tracheal tissue, and particularly macrophages contained greater amounts of unmetabolized 1-NP (Fig. 3).

DISCUSSION

It has been well documented that macrophages and respiratory tissues can metabolize carcinogenic polycyclic aromatic hydrocarbons into intermediates that bind DNA and other macromolecules (Harris *et al.*, 1978; Palmer *et al.*, 1978; Autrup *et al.*, 1978, 1979, 1980; Cohen *et al.*, 1979; Cohen and Moore, 1976, 1977).

In identical incubations of tracheal tissue, lung tissue, and macrophages, all were active in catalyzing the binding of ^{14}C-1-NP to endogenous DNA and protein; amounts of ^{14}C-1-NP–DNA binding in tracheal tissue were five times that observed in macrophages and lung tissue (King *et al.*, 1983a). The metabolism seen here in each tissue parallels the previously observed variations in binding levels; lung and more particularly tracheal tissue were more active than macrophages when compared on a per milligram of protein basis. A relatively small amount of the metabolites were retained by the macrophages and respiratory tissues, while the majority of the metabolites were released into the culture medium. Similarly, Palmer *et al.* (1978) reported that most of the metabolites of 7,12-dimethylbenz[*a*]anthracene (DMBA) formed by macrophages, lung tissues, and tracheal tissues were released into the culture medium. However, in that study, the macrophages retained more of the unmetabolized DMBA than did the other tissue; in our study, macrophages, lung tissues, and tracheal tissues incubated with 1-NP retained similar amounts of unmetabolized 1-NP. A similar substantially higher concentration of 1-NP metabolites was found in tracheal tissue in the present study. These findings are significant in view of the known susceptibility of rat and hamster tracheas to tumor induction by polycyclic hydrocarbons (Kuschner *et al.*, 1956; Saffioti *et al.*, 1968). Metabolites (α, 3-OH-1-NP, and β) isolated from lung S9 incubates were two to four times more mutagenic than the parent compound ^{14}C-1-NP in the Ames assay (King *et al.*, 1984). Phenolic metabolites formed by ring hydroxylation of 1-NP by the S9 fraction of the livers of Aroclor-induced rats have been shown to be mutagenic to *Salmonella typhimurium* TA98 and TA100 (El-Bayoumy and Hecht, 1983; Ball *et al.*,

1984a,b). The recently published results of Bond *et al.* (1984a) showed that small quantities of 10-OH-1-NP were detected in perfusates of rat lungs (<10% of the total metabolites).

The metabolite profiles obtained from macrophages and respiratory tissues suggest both nitro reduction (1-AMP, NAAP) and ring oxidation (phenols and dihydrodiols) and a degree of tissue specificity in the formation of metabolites.

ACKNOWLEDGMENTS

We thank Nathanette Mayo, Horace Tate, and James Scott for their excellent technical assistance. We also thank Karla Gunsolus of Northrop Services, Inc., for her editorial assistance.

REFERENCES

AUTRUP, H., HARRIS, C. C., SCHAFER, P. W., TRUMP, B. F., STONER, G. D., AND HSU, I. H. -C. (1979). Uptake of benzo[a]pyrene-ferric oxide particulates by human pulmonary macrophages and release of benzo[a]pyrene and its metabolites. *Proc. Soc. Exp. Biol. Med.* **161,** 280–284.

AUTRUP, H., HARRIS, C. C., STONER. G. D., SELKIRK, J. K., SCHAFER, P. W., AND TRUMP, B. F. (1978). Metabolism of [^{3}H]benzo[a]pyrene by cultured human bronchus and cultured human pulmonary alveolar macrophages. *Lab. Invest.* **38,** 217–223.

AUTRUP, H., WEFALD, F. C., JEFFREY, A. M., TATE, H., SCHWARTZ, R. D., TRUMP, B. F., AND HARRIS, C. C. (1980). Metabolism of benzo[a]pyrene by cultured tracheobronchial tissues from mice, rats, hamsters, bovines, and humans. *Int. J. Cancer* **25,** 293–300.

BALL, L. M., KOHAN, M. J., CLAXTON, L. D., AND LEWTAS, J. (1984a). Mutagenicity of derivatives and metabolites of 1-nitropyrene: Activation by rat liver S9 and bacterial enzymes. *Mutat. Res.* **138,** 113–125.

BALL, L. M., KOHAN, M. J., INMON, J. P., CLAXTON, L. D., AND LEWTAS, J. (1984b). Metabolism of 1-nitro[^{14}C]pyrene *in vivo* in the rat and mutagenicity of urinary metabolites. *Carcinogenesis* **5,** 1557–1564.

BOND, J. A., AND MAUDERLY, J. L. (1984a). Metabolism and macromolecular covalent binding of [^{14}C]-1-nitropyrene in isolated perfused and ventilated rat lungs. *Cancer Res.* **44,** 3924–3929.

BOND, J. A., MEDINSKY, M. A., AND DUTCHER, J. S. (1984b). Metabolism of 1-[^{14}C]nitropyrene in isolated perfused rat livers. *Toxicol. Appl. Pharmacol.* **75,** 531–538.

CAMPBELL, J., CRUMPLIN, G. C., GARNER, J. V., GARNER, R. C., MARTIN, C. N., AND RUTTER, A. (1981). Nitrated polycyclic aromatic hydrocarbons: Potent bacterial mutagens and stimulators of DNA repair synthesis in cultured human cells. *Carcinogenesis* **2,** 599–656.

CHAN, T. L., LEE, P. S., AND HERRING, W. E. (1981). Deposition and clearance of inhaled diesel exhaust particles in the respiratory tract of Fischer rats. *J. Appl. Toxicol.* **1,** 77–82.

CHEN, S., WELLER, M. A., AND BARNHART, M. I. (1980). *Effects of Diesel Engine Exhaust on Pulmonary Alveolar Macrophages.* Presented at the Scanning Electron Microscopy Meeting, SEM, Inc., AMF O'Hare, Chicago, Ill.

COHEN, G. M., MEHTA, R., AND BROWN, M. M. (1979). Large interindividual variations in metabolism of benzo(a)pyrene by peripheral lung tissue from lung cancer patients. *Int. J. Cancer* **24,** 129–133.

COHEN, G. M., AND MOORE, B. P. (1976). Metabolism of [^{3}H]benzo(a)pyrene by different portions of the respiratory tract. *Biochem. Pharmacol.* **25,** 1623–1629.

COHEN, G. M., AND MOORE, B. P. (1977). The metabolism of benzo(a)pyrene, 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene and 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene by short-term organ cultures of hamster lung. *Biochem. Pharmacol.* **26,** 1481–1482.

COLE, J., ARLETT, C. F., LOWE, J., AND BRIDGES, B. A. (1982). The mutagenic potency of 1,8-dinitropyrene in cultured mouse lymphoma cells. *Mutat. Res.* **93,** 213–220.

EL-BAYOUMY, K., AND HECHT, S. S. (1983). Identification and mutagenicity of metabolites of 1-nitropyrene formed by rat liver. *Cancer Res.* **43,** 3132–3137.

GARRETT, N. E., CAMPBELL, J. A., STACK, H. F., WATERS, M. D., AND LEWTAS, J. (1981). The utilization of the rabbit alveolar macrophage and Chinese hamster ovary cell for evaluation of the toxicity of particulate materials. 1. Model compounds and metal-coated fly ash. *Environ. Res.* **24,** 345–365.

HARRIS, C. C., HSU, I. C., STONER, G. D., TRUMP, B. F., AND SELKIRK, J. K. (1978). Human pulmonary alveolar macrophages metabolize benzo(a)pyrene to proximate and ultimate mutagens. *Nature (London)* **272,** 633–634.

HIRAO, F., FUJISAWA, T. E., AND YAMAMURA, Y. (1972). Experimental cancer of the lung in rabbits induced by chemical carcinogens. *Cancer Res.* **32,** 1209–1217.

HIROSE, M., LEE, M. S., WANG, C. Y., AND KING, C. M. (1984). Induction of rat mammary gland tumors by 1-nitropyrene, a recently recognized environmental mutagen. *Cancer Res.* **44,** 1158–1162.

HOWARD, P. C., FLAMMANG, T. J., AND BELAND, F. A. (1985). Comparison of the *in vitro* and *in vivo* hepatic metabolism of the carcinogen 1-nitropyrene. *Carcinogenesis* **6,** 243–249.

KING, L. C., JACKSON, M., BALL, L. M., AND LEWTAS, J. (1983a). Binding of 1-nitro[^{14}C]pyrene to DNA and protein in cultured lung macrophages and respiratory tissues. *Cancer Lett.* **19,** 241–246.

KING, L. C., KOHAN, M. J., BALL, L. M., AND LEWTAS,

J. (1984). Mutagenicity of 1-nitropyrene metabolites from lung S9. *Cancer Lett.* **22,** 255–262.

KING, L. C., LOUD, K., TEJADA, S. B., KOHAN, M. J., AND LEWTAS, J. (1983b). Evaluation of the release of mutagens and 1-nitropyrene from diesel particles in the presence of lung macrophage cells in culture. *Environ. Mutagen.* **5,** 577–588.

KUSCHNER, M. S., LASKIN, E. C., AND NELSON, N. (1956). Experimental carcinoma of the lung. In *Proceedings of the Third National Cancer Conference,* pp. 485–495. Lippincott, Philadelphia.

LEWTAS, J. (1982). Mutagenic activity of diesel emissions. In *Toxicological Effects of Emissions From Diesel Engines* (J. Lewtas, ed.), pp. 243–264. Elsevier, New York.

MCCLELLAN, R. O., BROOKS, A. L., CUDDIHY, R. G., JONES, R. K., MAUDERLY, J. L., AND WOLFF, R. K. (1982). Inhalation toxicology of diesel exhaust particles. In *Toxicological Effects of Emissions From Diesel Engines* (J. Lewtas, ed.), pp. 99–120. Elsevier, New York.

MERMELSTEIN, R., KIRIAZIDES, D. K., BUTLER, M., MCCOY, E. C., AND ROSENKRANZ, H. S. (1981). The extraordinary mutagenicity of nitropyrenes in bacteria. *Mutat. Res.* **89,** 187–196.

NESNOW, S., TRIPLETT, L. L., AND SLAGA, T. J. (1982). Comparative tumor initiating activity of complex mixtures of mouse skin. *J. Natl. Cancer Inst.* **68,** 829–834.

NISHIOKA, M. B., PETERSON, B., AND LEWTAS, J. (1982). Comparison of nitro-PNA content and mutagenicity of diesel emissions. In *Polynuclear Aromatic Hydrocarbons: Physical and Biological Chemistry* (M. Cook, A. J. Dennis, and G. L. Fisher, eds.), pp. 603–613. Battelle, Columbus, Ohio.

PALMER, W. G., ALLEN, T. J., AND TOMASZEWSKI, J. E. (1978). Metabolism of 7,12-dimethylbenz(a)anthracene by macrophages and uptake of macrophage-derived metabolites by respiratory tissues *in vitro. Cancer Res.* **38,** 1079–1084.

PETERSON, B. A., CHUANG, C. C., MARGARD, W. L., AND TRAYSER, D. A. (1981). Identification of mutagenic compounds in extracts of diesel exhaust particulates. *Air Pollut. Control Assoc. Abstract 74th Annual Meeting,* 81–56.1.

RUDD, C. J., AND STROM, K. A. (1981). Spectrophotometric method for the quantitation of diesel exhaust particles in guinea pig lung. *J. Appl. Toxicol.* **1,** 83–87.

SAFFIOTI, U., CEFIS, F., AND KOLB, L. H. (1968). A method for experimental induction of bronchogenic carcinoma. *Cancer Res.* **28,** 104–124.

SCHUETZLE, D. (1983). Sampling of vehicle emissions for chemical analysis and biological testing. *Environ. Health Perspect.* **47,** 65–80.

SCHUETZLE, D., LEE, F. S. C., AND PRATER, T. J. (1981). The identification of polynuclear aromatic hydrocarbon (PAH) derivatives in mutagenic fractions of diesel particulate extracts. *Int. J. Environ. Anal. Chem.* **9,** 93–144.

VOSTAL, J. J., SCHRECK, R. M., LEE, P. S., CHAN, T. L., AND SODERHOLM, S. C. (1982a). Deposition and clearance of diesel particles from the lung. In *Toxicological Effects of Emissions From Diesel Engines* (J. Lewtas, ed.), pp. 143–159. Elsevier, New York.

VOSTAL, J. J., WHITE, H. J., STROM, K. A., SIAK, J. S., CHEN, K. C., AND DZIEDZIC, D. (1982b). Response of the pulmonary defense system to diesel particulate exposure. In *Toxicological Effects of Emissions From Diesel Engines* (J. Lewtas, ed.), pp. 201–221. Elsevier, New York.

WHITE, H. J., AND GARG, B. D. (1982). Early pulmonary response of the rat lung to inhalation of high concentration of diesel particles. *J. Appl. Toxicol.* **1,** 104–110.

NOTICE

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

This article documents research that was conducted under the auspices of the U.S. Environmental Protection Agency (USEPA). As an arm of the government, the USEPA has a paid up, non-exclusive, irrevocable, worldwide license to reproduce, prepare derivative works, and distribute to the public all of the material contained herein, and to grant others acting on its behalf the right to do so. The USEPA grants to the National Technical Information Service, Department of Commerce, the right to act as its agent in the distribution of this material.